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Transgenic SOD1 G93A mice develop reduced GLT-1 in spinal cord without alterations in cerebrospinal fluid glutamate levels

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Abstract

Glutamate-induced excitotoxicity is suggested to play a central role in the development of amyotrophic lateral sclerosis (ALS), although it is still unclear whether it represents a primary cause in the cascade leading to motor neurone death. We used western blotting, immunocytochemistry and *in situ* hybridization to examine the expression of GLT-1 in transgenic mice carrying a mutated (G93A) human copper–zinc superoxide dismutase (TgSOD1 G93A), which closely mimic the features of ALS. We observed a progressive decrease in the immunoreactivity of the glial glutamate transporter (GLT-1) in the ventral, but not in the dorsal, horn of lumbar spinal cord. This effect was specifically found in 14- and 18-week-old mice that had motor function impairment, motor neurone loss and reactive astrocytosis. No changes in GLT-1 were observed at 8 weeks of age, before the appearance of clinical symptoms. Decreases in GLT-1 were accompanied by increased glial fibrillary acidic protein (GFAP) levels and no change in the levels of GLAST, another glial glutamate transporter. The glutamate concentration in the cerebrospinal fluid (CSF) of TgSOD1 G93A mice was not modified at any of the time points examined, compared with age-matched controls. These findings indicate that the loss of GLT-1 protein in ALS mice selectively occurs in the areas affected by neurodegeneration and reactive astrocytosis and it is not associated with increases of glutamate levels in CSF. The lack of changes in GLT-1 at the presymptomatic stage suggests that glial glutamate transporter reduction is not a primary event leading to motor neurone loss.

Keywords: excitotoxicity, glial fibrillary acidic protein, motor neurones, superoxide dismutase-1.

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Among the different hypotheses proposed so far for the aetiology of amyotrophic lateral sclerosis (ALS, motor neurone disease), glutamate-mediated excitotoxicity is proposed to play a major role in motor neurone degeneration (Shaw and Ince 1997). Abnormalities of glutamate metabolism, including increased glutamate concentrations in plasma and cerebrospinal fluid (CSF), have been documented in patients with the disease (Rothstein *et al.* 1990), although controversial results have been reported in this respect (Perry *et al.* 1990). More recently a selective loss of the major glutamate re-uptake transporter protein (EAAT2), was found in affected regions of the CNS of ALS patients (Rothstein *et al.* 1995). Decreased glutamate re-uptake into glial cells and/or neurones may result in increased extracellular glutamate levels that could allow excessive stimulation of glutamate receptors on motor neurones and therefore excitotoxic cell death.

However, at present, it is unknown whether alterations in the glutamate transporter and/or increase in the extracellular glutamate levels might be the initial mechanism which leads

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This paper is dedicated to the memory of Dr Rosario Samanin, Head of the Department of Neuroscience of the Mario Negri Institute, who sadly passed away while this manuscript was in preparation (June 4, 2001).

Abbreviations used: ALS, amyotrophic lateral sclerosis; CSF, cerebrospinal fluid; EAAT, excitatory aminoacid transporter; GFAP, glial fibrillary acidic protein; GLT-1 and GLAST, glial glutamate transporters; SOD1, copper–zinc superoxide dismutase.

to motor neurone damage or if these changes are secondary to cell death. These issues cannot easily be addressed from human studies, since examination in the CNS from ALS patients is generally done at the terminal stages of the disease when massive motor neurone loss has already occurred. Transgenic mice overexpressing mutations of copper–zinc superoxide dismutase (SOD1) have been generated, and provide reliable model to investigate the mechanisms underlying motor neurone degeneration. These animals develop progressive limb paralysis associated with spinal motor neurone degeneration, eventually leading to death (Gurney *et al.* 1994; Bruijn *et al.* 1997). It has been shown that the mutant SOD1 protein inactivates the GLT-1 in oocytes upon intra- or extracellular administration of H_2O_2 (Trotti *et al.* 1999). Thus, studies in mouse models of ALS at different stages of the disease progression could be used to determine whether alterations in glutamate transporter activity precede motor neurone degeneration.

Western blotting analysis of the total spinal cord extracts of transgenic mice carrying a G85R mutation of SOD1 suggested a decrease of the main glial glutamate transporter, EAAT-2 (also known in rodents as GLT-1), at the end stage of the disease (Bruijn *et al.* 1997). However, no quantitative analysis was provided in that study, and the study was not able to determine whether the decrease in GLT-1 occurred prior to the onset of the motor neurone pathology or after the disease was fully developed. Furthermore, no anatomical information was provided as to whether changes in glial glutamate transporters occurred in the more vulnerable regions such as the ventral horn of spinal cord. Studies on a different transgenic strain carrying a G93A mutation of SOD1 have shown decreased glutamate uptake into synaptosomes at the end stages of the disease (Canton *et al.* 1998), but since these studies examined neuronal uptake, it is not clear whether they also reflect alterations in glial glutamate transporter levels.

In order to determine whether glutamate abnormalities occur prior to or after the onset of the neuropathological features of ALS, we have used transgenic C57BL/6 mice expressing the human SOD1 with G93A mutation (TgSOD1 G93A). These mice show some morphological alterations of the motor neurones at 8 weeks of age, with an onset of motor symptoms and a significant motor neurone loss starting at 12–14 weeks and progressing to the end-stage symptoms of the disease at about 18–20 weeks (Migheli *et al.* 1999). The time-course of these symptoms differs from other transgenic strains, including TgSOD1G85R. We have used these TgSOD1 G93A mice to examine, at the different stages of the progression of the disease, the levels of glutamate in the plasma and CSF in fasting animals. We have also determined the relative levels of EAAT-2 and its mRNA in spinal cord using immunocytochemistry, *in situ* hybridization and western blotting, comparing the level to glial cell markers, GFAP or EAAT-1(GLAST).

Materials and methods

Materials

All materials were obtained from Sigma (Poole, UK) or Merck (Poole, UK) unless otherwise stated.

Animal model

Transgenic mice originally obtained from Jackson Laboratories and expressing high copy number of mutant human SOD1 with a Gly93-Ala substitution (TgSOD1 G93A) were bred and mantained on a C57BL/6 mice strain at the Consorzio Mario Negri Sud, S. Maria Imbaro (CH), Italy. Identification of transgenic mice was made by PCR (Rosen *et al.* 1993). The mice were maintained at a temperature of 21 ± 1°C with relative humidity 55 ± 10% and 12 h of light. Food (standard pellets) and water were supplied *ad libitum*. For the present study, female mice were killed at 8–10, 14 and 19 weeks of age corresponding, respectively, to a pre-symtomatic, symptomatic and late stage of the progression of the motor dysfunction (see below). Non-transgenic age-matched littermates were used as controls. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. no. 116, G.U. suppl. 40, Feb. 18, 1992, Circolare No. 8 , G.U., 14 luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1 DEC.12, 1987; NIH *Guide for the Care and Use of Laboratory Animals*, US National Research Council, 1996).

Glutamate determination in plasma and CSF

The mice were killed by decapitation after overnight fasting and the blood samples were collected in heparinized tubes and diluted 1 : 1 with 0.5% sodium dodecyl sulphate (SDS) solution, incubated for 10 min at room temperature (20 ± 5°C) and mixed 1 : 1.25 with 10% sulfosalicylic acid solution (v/v). Samples were vigorously mixed and centrifuged. Amino acid analysis was done on 50-µL supernatant fractions using a high performance amino acid analyser (AA Model 6300, Beckman Instrument Inc. Palo Alto, CA, USA), as previously described (Mennini *et al.* 1998). CSF samples were collected from the cisterna magna in Equithesin anaesthetized mice, after overnight fasting, using a glass microcapillary. Samples were rapidly frozen on dry ice. Glutamate was derivatized by mixing the samples with ortho-phthaldialdehyde/β-mercapto-ethanol (1 : 1). After 60 s reaction time the samples were injected onto the column. Peaks were detected using a WATER 470 fluorescence detector and quantified with a Spectra-Physics integrator. Data on glutamate levels in plasma and CSF were analysed by one-way ANOVA followed by Tukey's test for comparison between groups.

Western blot analyses of GLT-1 and GLAST

Mice (seven controls, seven TgSOD1 G93A mice at 10 weeks and six TgSOD1 G93A mice at 19 weeks) were killed by cervical dislocation and spinal cords removed and rapidly frozen. Samples of whole mouse spinal cord from control and transgenic animals were homogenised in protein lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.5 mM phenylmethylsulfonylfluoride, 10 µg/mL leupeptin, 10 µg/mL antipain, 1 µg/mL pepstatin, 1 µg/mL aprotinin, 1 mM Na_3VO_4, 50 mM NaF). Samples were centrifuged at 300 *g* at 4°C for 5 min to remove cell debris and then centrifuged at 35 000 *g* to obtain cytosol and membrane fractions. Protein assays were carried out

using the Bioquant reagent. Membrane (for GLT-1 or GLAST) or cytosolic (for actin) protein samples (20 μg) were mixed 4 : 1 with boiling buffer (50 mM Tris, pH 7.5, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue) and heated to 95°C for 5 min. Samples were separated by electrophoresis on denaturing polyacrylamide gels [8%, acrylamide: bis acrylamide (29 : 1), containing 0.1% SDS], and then electroblotted onto ECL Nitrocellulose membranes (Amersham Pharmacia Biotech, Little Chalfont, UK). Blots were incubated in 4% skimmed milk powder in TBS (20 mM Tris–HCL, 0.5 M NaCl, pH 7.5) for 30 min, washed twice for 5 min in TBS containing 0.05% Tween-20 (TTBS). Blots were then incubated overnight in TTBS containing antisera raised in rabbits against GLT-1 (aB12, 1 : 5000) or GLAST (aA522, 1 : 5000). Both antisera were a kind gift of Dr N. Danbolt (Lehre *et al.* 1995). As a control, mouse anti-actin antibody was used (JLA20, 1 : 1000). The monoclonal antibody developed by J. J. Lin (Lin 1981) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa (Department of Biological Sciences, Iowa City, IA, USA). Membranes were washed three times in TTBS and then incubated for 4 h in peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1 : 5000 in TTBS or peroxidase-conjugated goat anti-mouse IgG (Vector Laboratories, Peterborough, UK). Membranes were washed three times in TTBS, and proteins detected using the ECL plus detection system (Amersham Pharmacia Biotech), according to the manufacturer's instructions. Membranes were exposed to films, which were scanned and the band densities obtained using Bio Image Intelligent Quantifier (B.I. Systems, Ann Arbor, MI, USA). Data were analysed using one-way ANOVA with Tukey's *post hoc* tests as appropriate.

Immunohistochemistry

Mice (three controls for each age group, five TgSOD1 G93A mice at 8 or 18 weeks and three TgSOD1 G93A mice at 14 weeks) were anaesthetized with Equithesin (1% phenobarbitol/4% (v/v) chloral hydrate, 30 μL/10 g, i.p.) and transcardially perfused with 20 mL saline followed by 50-mL 4% paraformaldehyde in phosphate-buffered saline (PBS). Spinal cords were rapidly removed, post-fixed in fixative for 2 h, transferred to 20% sucrose in PBS overnight, then in 30% sucrose/PBS and finally frozen in 2-methylbutane at − 45°C. Sections (30 μm) were cut on a cryostat at − 20°C through the lumbar spinal cord in the transverse plane at the L_{2-4} level. Sections from each age group were processed at the same time using multiwell plates.

The sections were incubated in 10% normal goat serum in PBS for 1 h and kept overnight in the freshly prepared solution containing antibodies of GLT-1 (guinea pig polyclonal, 1 : 5000, Chemicon International, Temecula, CA, USA) or glial fibrillary acidic protein (GFAP mouse monoclonal, 1 : 250, Boehringher Mannheim). The next day, after washing in PBS, sections were incubated with biotin-conjugated secondary antibody for 1 h, washed and incubated in avidin–biotin–peroxidase (Vectastain kit, Vector Laboratories). After reacting with 3′-3-diaminobenzidine tetrahydrochloride sections were washed, mounted on poly lysine-coated slides, dried, dehydrated through graded alcohols, fixed in xylene and coverslipped using DPX mountant (BDH, Poole, UK) before light microscopic analysis. Control sections were incubated without the primary antibody. The optical density of GLT-1 immunostaining in the ventral and dorsal horn of lumbar spinal cord sections was measured relative to the medial dorsal white matter background of individual sections using an image analyser Imaging System KS300 (Zeiss–Kontron, Munchen, Germany). Optical density was measured within a linear range as determined by increasing dilution of the primary antibody. Ventral horn included the laminae VII, VIII and IX, and dorsal horn included the laminae I,II and III of the grey matter at the L3-L4 level of spinal cord. The optical densities of two sections were quantified for each animal and the mean value of these determinations was used as individual data for statistical analysis by ANOVA one way followed by Tukey's test.

In situ hybridization of GLT-1 mRNA

A rat GLT-1 cDNA fragment was prepared by PCR using standard methods (Molloy *et al.* 1998). A 327-bp fragment corresponding to bases 1310–1636 of the rat sequence (Pines *et al.* 1992) was amplified using specific primers (P3, AGCCGTGGCAGCC ATCTTCATAGC; M9, ATGTCTTCGTGCATTCGGTGTTGGG) and cloned into PCR2.1 (Invitrogen, Carlsbad, CA, USA). Riboprobe template was prepared by amplification of the plasmid (2 ng) in a volume of 500 μL with primers [PCR(T3)-P, ACCGAGCA ATTAACCCTCACTAAAGGGCCGCCAGTGTGCTGGAATTCG; PCR(M13F)-M, CGTTGTAAAACGACGGCC (AG)] that flanked the insert, and incorporated a T3 and T7 site into the template. GLT-1 template was purified using GFX columns (Amersham Pharmacia Biotech, UK).

Antisense and sense cRNA probes were synthesized by *in vitro* transcription from linear DNA templates (1 μg using, respectively, T7 or T3 RNA polymerase enzymes (Promega, Madison, WI, USA) in a reaction mixture containing transcription buffer 1×, dithiothreitol (DTT) 10 μM, RNase inhibitor 30 U (Boehringer Mannheim, Monza, Italy), non-labelled NTP 0,5 mM and UTP 10 μM (Promega) and [^{35}S]UTP 50 mCi (Amersham Pharmacia Biotech). Unincorporated nucleotides were separated from probes using a Quick spin column G50 Sephadex (Boehringer Mannheim), then probes were degraded to 150 base fragments by alkaline hydrolysis. Following ethanol precipitation and denaturation (80°C for 5 min), probes were diluted to 1.5 × 10^4 cpm/μL with hybridization buffer containing 50% formamide, 2 × sodium saline citrate buffer (SSC), 10 mM Tris–HCl pH 7,5, Denhart's solution 1×, dextran sulfate 10%, 0.2% SDS, 100 mM DTT, 500 μg/mL double-strand Salmon Sperm DNA and 250 mg/mL yeast tRNA.

Spinal cord sections (14 μm) of TgSOD1 G93A mice and controls at different stages of disease progression were cut using a cryostat, and mounted on poly L-lysine-coated microscope slides. The slides were then rapidly immersed in 4% paraformaldehyde freshly prepared in 0.1 M PBS for 5 min, rinsed twice in PBS, acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl pH 8 for 10 min, dehydrated through a graded series of ethanol and delipidated in chloroform. Sections were then air-dried and stored frozen at − 70°C until the day of experiment. At the day of the experiment, the slides were brought to room temperature. Then the ^{35}S-radiolabelled riboprobe was applied to each slide and the slides were coverslipped with parafilm and incubated overnight at 42°C.

After hybridization, slides were washed in 2 × SSC at room temperature for 1 h and 2 × SSC at 60°C for 1 h. All slides were

treated with RNase A at 37°C for 30 min (20 μg/mL RNase A in 0.5 M NaCl, 10 mM Tris–HCl pH 7.5, 1 mM EDTA) and washed in 0.1% SSC at 60°C for 1 h. All washing solution contained 10 mM β-mercaptoethanol. Sections were dehydrated through a graded series of ethanols, each containing 0.3 M ammonium acetate, and then air-dried. Slides were exposed to X-ray film (Biomax MR; Kodak, Rochester, NY, USA) for 21 days. Films were developed by X-ray developer, washed and fixed by X-ray fixer (Kodak), then air-dried before optical density quantification using a computerized image analyser KS 300 (Zeiss-Kontron). Optical density was measured within the linear range as determined by using ^{35}S-brain paste standards. For each animals, two spinal cord sections were analysed and the mean of the two values was used for statistical analysis by one-way ANOVA followed by Tukey's test.

Results

Behavioural and histological analysis of mice

The transgenic strain used showed noticeable signs of neuromuscular dysfunction such as limb tremors, deficit in the hindlimbs extension reflex and initial impairment in motor behaviour starting at about 12–14 weeks of age. At 19–20 weeks, all the TgSOD1 G93A mice showed a marked difficulty in the performance of all motor tasks which was associated with paralysis and muscular atrophy of the hind limbs and they died by 142 ± 11 (SD) days. Histologically they show abnormalities in the motor neurones and axons, i.e. vacuolization of the cytoplasm and mitochondria, at about 8 weeks of age, before the symptoms become evident while by 12–14 weeks a progressive loss of motor neurones occurs in the lumbar spinal cord amounting to 44 ± 3% and 52 ± 2% at 12 and 18 weeks, respectively. The number of motor neurones of 8-week-old TgSOD1 G93A mice was unchanged compared with age-matched controls.

For the present study, female mice were killed at 8–10, 14 and 18–19 weeks of age corresponding, respectively, to a presymtomatic, symptomatic and late stage of the progression of the motor dysfunction. Non-transgenic age-matched littermates were used as controls.

GLT-1 and GLAST immunoblotting

Using western blotting to analyse proteins in the size range 35–100 kDa, each of the antibodies recognized a single band of molecular weight 66 kDa for GLT-1, 67 kDa for GLAST and 43 kDa for actin, in line with previously reported data (Lehre *et al.* 1995; Akbar *et al.* 1998) (Fig. 1). The levels of GLT-1 appeared to be decreased in transgenic mice, compared with controls whilst the levels of another glial glutamate transporter, GLAST, were relatively unchanged. Analysis of the data revealed that the levels of GLT-1 tended to be decreased compared with controls, either when the raw data was considered (not shown) or when GLT-1 levels were expressed relative to the amount of actin from the same tissue (Table 1). When compared with level of GLAST in the same samples (Table 1), the level of

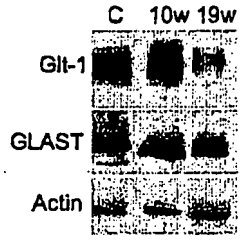

Fig. 1 Representative examples of western blotting of spinal cord protein extracts probed for GLT-1, GLAST and actin. Lanes show samples from control tissue (C), and transgenic mice at 10 and 19 weeks of age. GLT-1, GLAST and actin band sizes were 66 kDa, 67 kDa and 43 Da, respectively. Decreases in GLT-1 in transgenic mice are apparent, without reductions in actin or GLAST.

GLT-1 relative to GLAST was significantly decreased in transgenic mice at 19 weeks, but not 10 weeks, compared with controls (Table 1).

In view of the existence of multimeric complexes of GLT-1 and that oxidative damage, such as might occur during the development of motor neurone disease, might increase the presence of these complexes (Haugeto *et al.* 1996; Trotti *et al.* 1998), samples were analysed in more detail for GLT-1 protein using western blotting. Blots were prepared to analyse bands over a much wider size range (Fig. 2). The results showed that as well as the prominent band of *c.* 66 kDa representing monomeric GLT-1, a larger molecular weight band (*c.* 200 kDa) was present in all the samples, most likely representing a trimeric complex of GLT-1 (Haugeto *et al.* 1996). No bands were found at intermediate molecular weights representing dimeric GLT-1. In addition, for some samples, a lower molecular weight band was found (*c.* 45 kDa), possibly representing a degradation product of GLT-1; the presence or absence of this band did not relate to the disease state.

Quantification of the higher molecular weight (200 kDa) band revealed that there was no increase in TgSOD1 G93A

Table 1 Glutamate transporter levels in spinal cord

	GLT1/actin	GLAST/actin	GLT1/GLAST
Controls	1.54 ± 0.58	1.34 ± 0.41	1.31 ± 0.37
G93A 10-week-old	2.14 ± 0.74	1.22 ± 0.21	2.13 ± 0.78
G93A 19-week-old	0.68 ± 0.29	1.25 ± 0.27	0.70 ± 0.22*

Western blotting was used to determine levels of GLT-1, GLAST and actin in the lumbar spinal cord from control animals ($n = 7$), presymptomatic transgenic animals (G93A 10-week-old, $n = 7$), and end-stage transgenic animals (G93A 19-week-old, $n = 6$). Data were analysed by one-way ANOVA followed by Tukey's test, where appropriate. *$p < 0.05$ compared with controls.

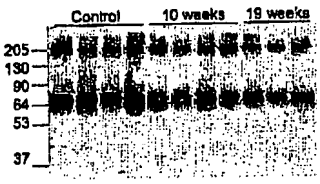

Fig. 2 Western blotting of spinal cord protein extracts probed for GLT-1. Lanes show samples from control tissue and transgenic mice at 10 and 19 weeks of age. GLT-1 immunoreactive bands were found at c. 68 kDa, corresponding to monomeric protein and c. 200 kDa, corresponding to a multimer and a band of lower molecular weight (c. 45 kDa) in some samples. Note that levels of immunoreactivity for the 68-kDa and 200-kDa bands were lower in transgenic mice compared with controls.

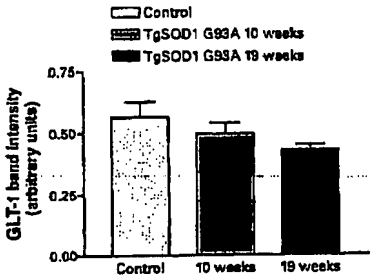

Fig. 3 Quantitative analysis of western blots of spinal cord extracts probed for GLT-1, analysing the intensities of the 200-kDa heteromultimeric band. Band intensities are in arbitrary units. The histograms represent the mean values of four control animals, four TgSOD1 G93A at 10 weeks and three TgSOD1 G93A at 19 weeks.

mice at any of the time points studied; in fact, the levels of the 200 kDa homomultimer were decreased slightly in transgenic mice compared with controls (Fig. 3), as were the levels of GLT-1 monomers.

As the decrease in whole spinal cord GLT-1 was relatively small, pilot experiments of immunoblotting were done separating the dorsal and ventral region of the spinal cord; however, the poor reproducibility of the tissue dissection produced high variability of the samples. Therefore a more detailed analysis of the change in GLT-1 was carried out using immunocytochemistry and *in situ* hybridization, that allowed anatomical resolution of the changes to particular regions of the spinal cord and to glial cells.

GLT-1 immunohistochemistry

In control mice, lumbar spinal cord the GLT-1 immunoreactivity is distributed diffusely throughout the grey matter with the highest immunoreactivity observed in the substantia gelatinosa of the dorsal horn. The white matter shows a filamentous pattern of strong immunostaining in the ventral and lateral regions of the spinal cord while a low signal appears in the dorsomedial region (Fig. 4). This agrees with the distribution described in the rat and human spinal cord, where GLT-1 immunoreactivity has been reported to be exclusively of glial origin (Rothstein *et al.* 1994; Fray *et al.* 1998).

Compared with age-matched controls, a marked, and statistically significant decrease in the GLT-1 immunostaining in the ventral horn of the spinal cord is evident in the TgSOD1 G93A mice at 14 and 18 weeks of age, where motor impairments were present. However, there was no reduction in GLT-1 in presymptomatic animals at 8 weeks of age compared with non-transgenic age-matched mice. No significant changes are observed in the dorsal horn of TgSOD1 G93A mice at any of the ages tested (Fig. 5).

GLT-1 *in situ* hybridization

The riboprobe for GLT-1 revealed widespread distribution of this mRNA thoughout the grey matter of the spinal cord (Fig. 6a), consistent with the expression of this mRNA in astrocytes. Analysis of data showed no change in the levels of GLT-1 mRNA in either the ventral or dorsal regions of the spinal cord of TgSOD1 G93A mice compared with controls (Fig. 6b).

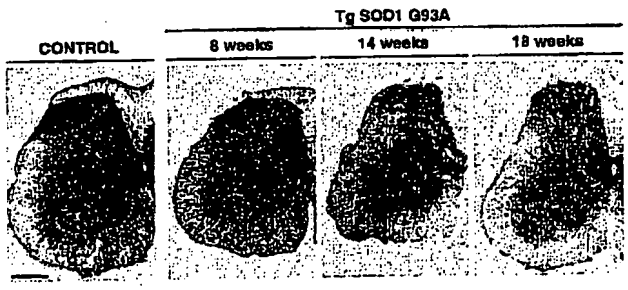

Fig. 4 GLT-1 immunoreactivity in the representative semisections of the lumbar spinal cord of mice. In non-transgenic mice, GLT-1 immunostaining is mainly distributed throughout the grey matter with the highest signal shown at the level of the substantia gelatinosa of dorsal horn. Note a gradual decrease of GLT-1 immunostaining in the ventral and intermediate region of the grey matter in TgSOD1 G93A mice at 14 and 18 weeks of age, while the dorsal horn maintains high immunostaining in TgSOD1 G93A mice at all ages compared with controls. Bar = 250 μm.

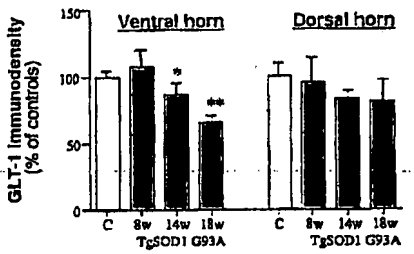

Fig. 5 Quantitative analysis of the GLT-1 immunostaining in the ventral and dorsal horn of the spinal cord. Data are expressed as percent of control. The optical densities of two sections were quantified for each animal ($n = 3$–5) and the mean value of these determinations was used as individual data for statistical analysis by one-way ANOVA followed by Tukey's test. $^{*}p < 0.05$; $^{**}p < 0.01$ compared with controls (C; Tukey's test).

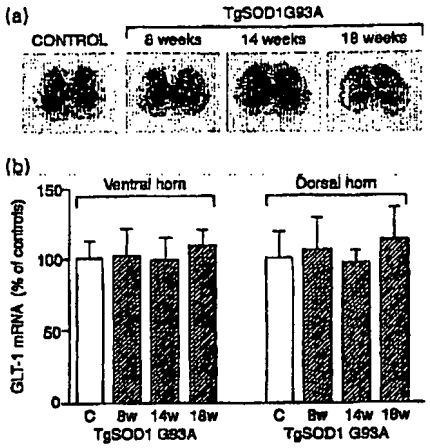

Fig. 6 (a) GLT-1 mRNA in spinal cord determined by *in situ* hybridization. GLT-1 mRNA is found throughout the spinal cord, mainly in the grey matter. There are no clear differences between control and transgenic animals. (b) Quantitative analysis of GLT-1 mRNA in the ventral and dorsal horn of the spinal cord. Data are expressed as percent of control. The optical densities of two sections were quantified for each animal and the mean value of these determinations was used as individual data for statistical analysis by ANOVA one way followed by Tukey's test.

GFAP immunohistochemistry

In the lumbar spinal cord of control mice, the GFAP immunostaining shows a scattered distribution in the grey matter, whereas in the white matter it appears in a filamentous pattern. (Fig. 7). At high magnification, the astrocytes appear with the characteristic stellar shape with a tiny cell body surrounded by branched thin processes (Fig. 7a). In TgSOD1 G93A mice, a progressive increase of GFAP immunolabelling occurs from 8 weeks of age; at the advanced stages (14 and 18 weeks) several astrocytes also show a remarkable hypertrophy of the cell bodies and processes mainly in the ventral region of the spinal cord (Figs 7b–d).

Glutamate levels in CSF and plasma

The mean levels of glutamate in plasma or CSF from all controls were, respectively, 113.2 ± 13.2 and 6.2 ± 0.9 nmol/mL (mean ± SEM). We observed no significant changes in the CSF glutamate concentrations of TgSOD1 G93A mice at 14 and 18 weeks of age in respect to age-matched control mice (Fig. 8). No significant differences were also observed in the plasma levels of glutamate in TgSOD1 G93A mice at 8 and 14 weeks compared with age-matched controls, whereas at 18 weeks the levels were significantly lower (Fig. 8).

Discussion

In this study we have carried out a detailed characterization of the time-course of changes in glutamate, and the main glutamate transporter protein GLT-1 (EAAT-2), in SOD1 G93A transgenic mice. We have focussed on the spinal cord, where the main loss of motor neurones is found. Our findings reveal that there are changes in glial cell markers: the glutamate transporter GLT-1 is decreased, the glial cytoskeletal marker, GFAP, is increased and GLAST is unchanged. The decrease in glutamate transporter levels do not result in a measurable change in CSF glutamate level. The biggest changes are in the most vulnerable regions of spinal cord, and are found at end stages of the disease.

Time course and regional selectivity of GLT-1 reduction

The present study reveals that levels of the protein GLT-1 (EAAT-2), but not its mRNA, are significantly decreased in the ventral grey region of lumbar spinal cord of TgSOD1 G93A mice at the advanced, but not at the presymptomatic, stage of the disease. Using immunocytochemistry and western blotting, we found changes in GLT-1 in transgenic animals in the whole spinal cord at 19 weeks, and changes in GLT-1 in the ventral horn of the spinal cord at 14 and 18 weeks. At these time points, there is marked motor neurone death and clear symptoms of the disease. These results are consistent with previous observations on a similar transgenic strain, which showed that synaptosomal glutamate uptake was not decreased between 8 and 17 weeks in transgenic mice, but was significantly reduced at 21 weeks (Canton *et al.* 1998). The present results suggest that alterations in glial expression of glutamate transporter expression occur, but only at the late stages of the disease.

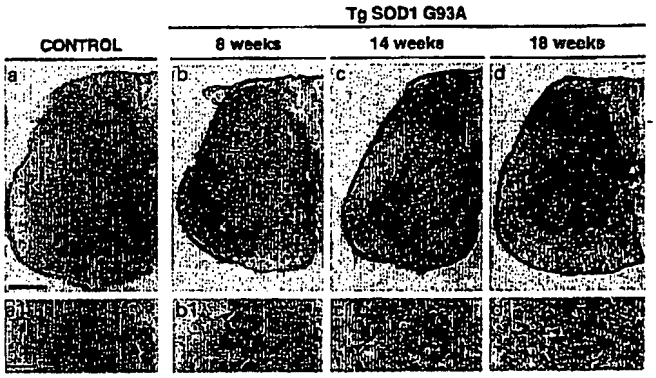

Fig. 7 GFAP immunoreactivity in the lumbar spinal cord of mice. In non-transgenic mice, GFAP immunostaining is scattered distributed in the grey matter with a filamentous pattern in the white matter. In (a–d) note a gradual increase of the GFAP immunostaining in the whole section of the spinal cord in TgSOD1 G93A mice at the different ages with a prominent increase in the grey matter of 14- and 18-week-old mice. (a1–d1) High magnification of the astrocytes from the ventral horn region shows in control mice a characteristic stellar shape with a tiny cell body surrounded by branched thin processes (arrows), whereas in TgSOD1 G93A mice, the astrocytes appeared more intensely stained with hypertrophic cell bodies and processes, particularly in 18-week-old mice. Bar = 250 μm (a–d) and 50 μm (a1–b1).

At 8 weeks of age these transgenic mice do not show motor neurone loss or clinical symptoms although morphological alterations of motor neurones are apparent, for example widespread vacuolization of cytoplasm and swelling of mitochondria (Migheli *et al.* 1999; Bendotti *et al.* 2001). As reported here, initial signs of reactive astrocytosis, as demonstrated by enhanced immunolabelling for GFAP, are also found at this age. Therefore, the present results suggest that the early stage of motor neurone damage in TgSOD1 G93A mice is not associated with changes in GLT-1 protein levels.

Increasing evidence indicates that glutamate transporters, and in particular GLT-1, are vulnerable to the oxidation resulting in an impaired glutamate uptake function (Trotti *et al.* 1998). In particular, it has been shown that the mutant SOD1 protein inactivates the GLT-1 in oocytes upon intra- or extracellular administration of H_2O_2 (Trotti *et al.* 1999). Thus, although our study demonstrates that the level of GLT-1 is unchanged in the lumbar spinal cord of young transgenic mice, we cannot exclude that the activity of this transporter is already compromised at this age. This may play a role in the initial process of motor neuronal death by mediating an increase in extracellular glutamate concentration. Interestingly, recent studies (Alexander *et al.* 2000; Andreassen *et al.* 2001) showed, by intracerebral microdialysis, an increase of extracellular fluid glutamate in somatosensory cortex of TgSOD1 G93A mice, at the advanced stage of the disease, without visible pathology in this brain region. This effect was magnified by the perfusion with a glutamate uptake inhibitor or by the NMDA glutamate receptor stimulation, whereas it was significantly attenuated by creatine treatment which also increased survival and motor performance of TgSOD1 G93A mice (Andreassen *et al.* 2001). This effect was interpreted as a decreased capacity to clear glutamate from the extracellular space although the cortical GLT-1 levels measured by immunoblotting appeared unchanged (Alexander *et al.* 2000). It has been reported that oxidative conditions may induce the formation of GLT-1 homomultimers, thus reducing the amount of monomer revealed by immunoblotting (Haugeto *et al.* 1996), and evidence suggests that oligomeric structure of GLT-1 is required for transport activity. Although there is substantial evidence for oxidative damage in transgenic mice with G93A SOD1 mutation (Andrus *et al.* 1998), we did not observe an increase but rather a slight decrease in the homomultimeric form in these mice at the advanced stage of the disease. This indicates that oxidative damage which might lead to the inactivation of

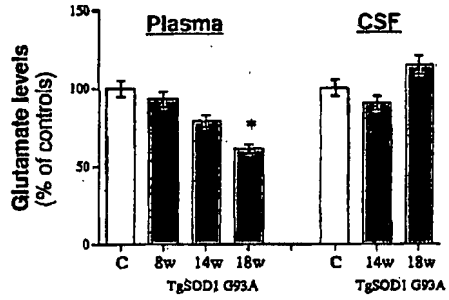

Fig. 8 Glutamate in CSF and plasma from transgenic mice. Concentrations of glutamate in plasma and cerebrospinal fluid (CSF) from TgSOD1 G93A mice at different ages compared with controls (C; white bars). Values are expressed in percent of age-matched non-transgenic controls. Each bar represents the mean ± SE. *$p < 0.05$ compared with respective controls (one-way ANOVA).

GLT-1 in TgSOD1 G93A mice at the early stage, might, in the long-term, reduce the transporter levels.

Our study demonstrates that the decrease of GLT-1 specifically occurs in the regions affected by the pathology since no significant changes in immunoreactivity were observed in the dorsal horn of the spinal cord where there is no neuronal degeneration. Our results are in line with those obtained from post-mortem tissues of patients with sporadic and familial ALS showing a selective decrease of GLT-1 in spinal cord and motor cortex but not in other unaffected brain regions (Rothstein *et al.* 1995). More recently, Fray *et al.* (1998) using quantitative EAAT2 immunohistochemistry, have shown a decrease in all regions of the lumbar spinal cords from motor neurone disease patients, although the highest reduction was observed at the level of the ventral horn and intermediate grey, the most affected areas. Moreover, as found in ALS patients (Bristol and Rothstein 1996), we have not found changes in the levels of mRNA of GLT-1 in the lumbar spinal cord of SOD1 G93A at any stage of the disease. Thus, these results suggest that the decrease of GLT-1 is due to a post-transcriptional regulation of this protein.

Alterations in GLT-1 compared with other glial proteins, GLAST and GFAP

The changes in GLT-1 seem to be specifically associated with disease progression, compared with the other glutamate transporter studied. We observed by quantitative immunoblotting a decrease of the GLT-1 but not GLAST in the lumbar spinal cord extract of SOD1 G93A mice during the progression of the disease, suggesting a differential regulation of these two glial glutamate transporters under these pathological conditions. Since it has been reported that the expression of glutamate transporter subtypes in astrocytes is under neuronal regulation (Gegelashvili *et al.* 1997; Swanson *et al.* 1997; Perego *et al.* 2000), we suggest that the decrease in GLT-1 observed in the transgenic FALS mice, as well as in the post-mortem tissue of patients with ALS, results from a loss of neuronal factors regulating the expression of this glial glutamate transporter in the vulnerable regions. The fact that GLAST was not changed suggests that these two glial transporters in the spinal cord may be regulated differently by neuronal factors. This is in line with studies in cultures of cortical astrocytes that showed that the expression of GLT-1 is much more dependent on neuronal factors and intact neurones compared with GLAST (Gegelashvili *et al.* 1997).

The loss of GLT-1 occurred in the spinal cord of motor impaired mice in the presence of strongly activated astrocytes in this region as demonstrated by their hypertrophy and increased immunostaining of GFAP. This agrees with a previous study of Mennini *et al.* (1998) showing, by immunoblotting, a significant decrease of GLT-1 associated with an increase in GFAP in the spinal cord of *mnd* mice, a strain which develops neuronal ceroid lipofuscinosis with a late-onset paralysis of hind limbs (Bronson *et al.* 1993). Moreover, a down-regulation of GLT-1 together with an increase in GFAP was reported in rat brain following lesion of corticostriatal pathway (Levy *et al.* 1995). This suggests that hypertrophic astrocytes show an altered functional activity which may induce a down-regulation of the GLT-1, but not GLAST, glutamate transporter.

Glutamate transporters and CSF glutamate levels

In this study, we did not find any increase in the levels of glutamate in the CSF of transgenic mice. Our findings differ from observations in ALS patients which showed increased CSF levels of glutamate (Rothstein *et al.* 1990). Nevertheless, controversial evidence exists in this respect (Perry *et al.* 1990).

CSF concentration of glutamate reflects multiple processes including release and uptake, as well as glutamate metabolism. In this regard, decreased activity of glutamate dehydrogenase (GDH), an enzyme playing a central role in glutamate metabolism, has been suggested to be responsible of the increased levels of glutamate in the CSF and plasma of ALS patients (Plaitakis 1990), although a down-regulation of EAAT2 may also contribute to this effect. The lack of increase in the levels of glutamate in the CSF and plasma of transgenic mice therefore suggests that the GLT-1 reduction observed in the ventral spinal cord of these mice does not significantly modify the total pool of extracellular glutamate. We cannot exclude the possibility that an increase in the extracellular concentration of this amino acid, with functional relevance for the neurodegenerative process, may occur in the vicinity of motor neurones in the ventral spinal cord of TgSOD1 G93A mice as consequence of GLT-1 reduction.

The decrease in plasma glutamate levels observed at the late stage of the disease is likely to be consequent to muscle loss in these mice reducing the pool of metabolic glutamate. A similar decrease in plasma glutamate levels has been found in *mnd* mice at the advanced stage of the disease, although at the stage preceding the onset of neuromuscular deficit they showed increased plasma concentration of glutamate (Mennini *et al.* 1998).

Conclusion

In conclusion, this study clearly demonstrates a selective GLT-1 decrease in the spinal cord of TgSOD1 G93A mice which is likely due to a loss of neuronal factors following motor neurone death and/or to a sustained long-lasting oxidative damage of the protein. This effect therefore is not a primary event leading to motor neurone degeneration but may contribute to the rapid progression of the disease at the advanced stage.

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